Recent advances in the molecular genetics of resin biosynthesis and genetic engineering strategies to improve defenses in conifers

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Abstract: Since the first terpenoid synthase cDNA was obtained by the reverse genetic approach from grand fir, great progress in the molecular genetics of terpenoid formation has been made with angiosperms and genes encoding a monoterpene synthase, a sesquiterpene synthase, and a diterpene synthase. Tree killing bark beetles and their vectored fungal pathogens are the most destructive agents of conifer forests worldwide. Conifers defend against attack by the constitutive and inducible production of oleoresin that accumulates at the wound site to kill invaders and both flush and seal the injury. Although toxic to the bark beetle and fungal pathogen, oleoresin also plays a central role in the chemical ecology of these boring insects. Recent advances in the molecular genetics of terpenoid biosynthesis provide evidence for the evolutionary origins of oleoresin and permit consideration of genetic engineering strategies to improve conifer defenses as a component of modern forest biotechnology. This review described enzymes of resin biosynthesis, structural feathers of genes genomic intron and exon organization, pathway organization and evolution, resin production and accumulation, interactions between conifer and bark beetle, and engineering strategies to improve conifer defenses.

Keywords: Genetic engineering strategies; Resin biosynthesis; Bark beetles; Genomics; Molecular genetics

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Introduction

Conifers are a very important group of woody plants in the world, especially in Western North America, Eastern Asia, and parts of Australia and New Zealand (Scagel *et al.* 1965). There are 600 species of conifers in seven families including *Pinaceae, Podocarpaceae, Araucariaceae, Taxodiaceae, Cephalotaxaceae, Taxaceae, Cupressaceae* (Gifford and Foster 1998), which dominate large regions of temperate forest. The *Pinaceae* is the most abundant and widespread of these families. Conifers are subject to predation by a wide range of herbivores and pathogens with the most serious destruction worldwide resulting from the infestation by tree killing bark beetles and their symbiotic fungal pathogens. A principal and unique defense of conifers is comprised of the constitutive and inducible production of oleoresin (often termed resin or pitch) (Mutton 1962). Accumulated resin is released upon tissue injury or produced locally at the site of infestation, with the consequence that the beetle and associated fungal pathogens are killed or encased in resin. This process is called pitching out, and it results in moving the oleoresin to the trunk surface where the turpentine evaporates to permit the resin acids to form a formidable physical barrier that seals the wound (Gijzen et al. 1993). With the advent of large-scale commercial lumbering of softwoods and the replanting of clear cuts with a single conifer species, predation by bark beetles has become a serious problem in mono-cultural forestry. For these reasons, the biology of bark beetles and their attack strategies and dynamics have become the focus of considerable study. The response of the host conifer and the physical and chemical character of the defensive secretion have also been subjected to substantial investigation. Much recent work has focused on a more refined analysis of conifer resin production. The evolutionary origins of oleoresin are briefly described, as are the prospects for biotechnological applications using these newer molecular tools. There are very few traditional pest control strategies applicable to large-scale forestry.

Molecular genetics of resin biosynthesis

Oleoresin (resin) terpenes arise from the fundamental precursor isopentenyl diphosphate (Figure 1 and 2) at one of two subcellular locations in plants. Following production of isopentenyl diphosphate and its isomerization to dimethylallyl diphosphate by isopentenyl diphosphate isomerase, the latter is condensed with one, two, or three units of isopentenyl diphosphate by specific prenyltransferases at the corresponding subcellular locales to give the respective precursors of the monoterpenes (geranyl diphosphate), sesquiterpenes (farnesyl diphosphate), and diterpenes (geranylgeranyl diphosphate) (Figure 3). The terpenoid synthases next convert the respective acyclic precursors, geranyl, farnesyl, and geranylgeranyl diphosphate, to the various parent structural derivatives of the different terpene families and so represent the committed enzymes of these pathways (Davis and Croteau 2000). These enzymes are

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often called cyclases because most of the products derived from the three central acyclic precursors are cyclic. Terpenoid synthases have been isolated and characterized from several conifer species, but the bulk of this work has been carried out with grand fir (*A. grandis*), a common and widespread species in the Pacific Northwest. The plant is easily raised in the greenhouse, and oleoresin production is wound-inducible, which mimics bark beetle attack (Lewinsohn et al. 1992), thereby offering significant advantage for biochemical and molecular study.

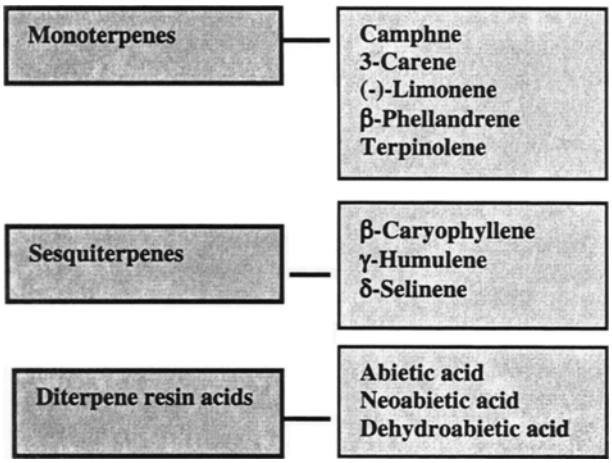

Fig. 1 Structures of typical resin components.

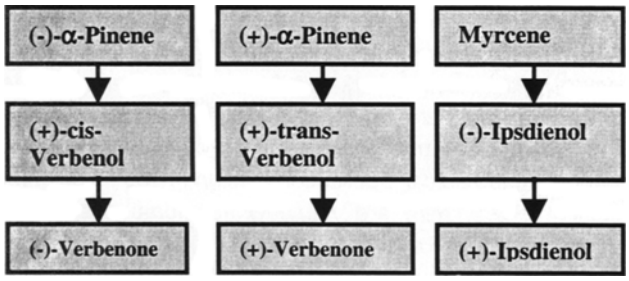

Fig. 2 Pheromone biosynthesis.

The structures of selected insect pheromones and their corresponding monoterpene olefin precursors are shown. Transformations occur typically in the hindgut of the bark beetle and have been attributed to associate microorganisms. Depending on the species, pheromones may signal aggregation or dispersal, and they may also attract beetle predators and parasitoids (Gijzen et al. 1993).

The monoterpene synthases are very similar to each other (Gijzen *et al.* 1991) and differ little in their properties from their counterparts isolated from pine species. They require a divalent metal ion for catalysis (Mg^{2+}, Mn^{2+}, or Fe^{2+}) (Savage *et al.* 1994). All conifer monoterpene synthases appear to arise as preproteins targeted to the plastids and produce acyclic or cyclic olefins as products (Wise and Croteau 1999). Host monoterpene olefins do serve as the precursors of several oxygenated derivatives produced and employed by bark beetles as pheromones (Fig. 3). The monoterpene synthase reaction involves the initial, metal ion–assisted ionization of the substrate to the corresponding carbocation-diphosphate anion pair, which may undergo direct deprotonation at various positions to yield the acyclic monoterpenes, such as myrcene. Many monoterpene synthases essentially produce a single product, but there are several examples of these enzymes that produce multiple products from the geranyl substrate (Savage *et al.* 1994). The similarity in properties, and also in primary sequence, of these enzymes indicates that rather subtle differences in structure and mechanism can result in very different product outcomes. Sesquiterpene synthases from conifers are cytosolic enzymes and resemble the plastidial monoterpene synthases in general properties, although the divalent cation requirement may differ and monovalent cations have no influence on reaction rate (Phillips and Croteau 1999). The various olefin isomers, differing in the substituents on the third ring and in the positions of the double bonds, undergo oxidative conversion of the C^{18} methyl to a carboxyl to yield the corresponding resin acid (LaFever *et al.* 1994). In the case of abietic acid, one of the most common resin acids and the most prone to polymerization, the abietadiene precursor is oxidized in three distinguishable steps to the resin acid by two cytochrome P450 hydroxylases and a dehydrogenase (Funk and Croteau 1994).

The grand fir has been developed as a model for studying the regulation of conifer defense because of the wound-inducibility of oleoresin production in saplings. The time course of the response was initially investigated at the level of resin chemistry by monitoring the levels of the relevant monoterpene, sesquiterpene, and diterpene biosynthetic enzymes (Steele *et al.* 1995). The production of induced oleoresin monoterpenes, sesquiterpenes, and diterpenes is coordinated to accomplish a range of defense goals. The turpentine components provide the principal toxins (Raffa *et al.* 1985) and solvent for mobilization of the resin acids. The cytochrome P450 oxygenases involved in resin acid biosynthesis are up-regulated in coordination with the terpene synthases (Funk *et al.* 1994). Given the complexity of the bark beetle-host interaction, and the many functions of the oleoresin, it is not a trivial exercise to consider what a chemically and physically optimized resin should contain. Little guidance is provided by an assessment of the induced response among a native population of grand fir in which investigators observed wide variation (Katon and Croteau 1998). This evidence suggests that there is no single best strategy to deter beetle attack. Studies with water-stressed and light-stressed trees have demonstrated that such stresses delay or diminish the response (Lewinsohn *et al.* 1993). These results are consistent with field observations indicating that physiologically compromised trees are most readily overwhelmed by massed beetle attacks and often provide the focus for wider spread infestation. With the availability of cDNA clones that encode several of the monoterpene, sesquiter-

pene, and diterpene synthases of grand fir, researchers are able to examine the defense response in greater detail and demonstrate that induced oleoresinosis arises via the differential transcriptional control of the synthase genes in response to wounding. RNA-blot hybridization analysis using terpene synthase class-specific probes indicates that the monoterpene synthases arise first and are followed by a coordinated increase in sesquiterpene and diterpene synthase transcripts (Steele *et al.* 1998). This dynamic alteration is consistent with the notion that the production of toxic monoterpenes is first upregulated, then followed by the production of monoterpenes that are more notable for their solvent properties in dissolving resin acids. The time delay in production of diterpene resin acids following the initiation of monoterpene biosynthesis might result from the need to first generate the solvent for rosin mobilization, and it fits the time-frame of beetle attack (Phillips and Croteau 1999). Although the sesquiterpenes make up only a small portion of conifer oleoresin, the mixture of constitutive sesquiterpenes is exceedingly complex (Bohlmann *et al.* 1998). Researchers have shown the wound induced accumulation of juvenile hormone analogs in intact fir (Puritch and Nijholt 1974), but they have not evaluated the direct influence of this phenomenon on insect fecundity.

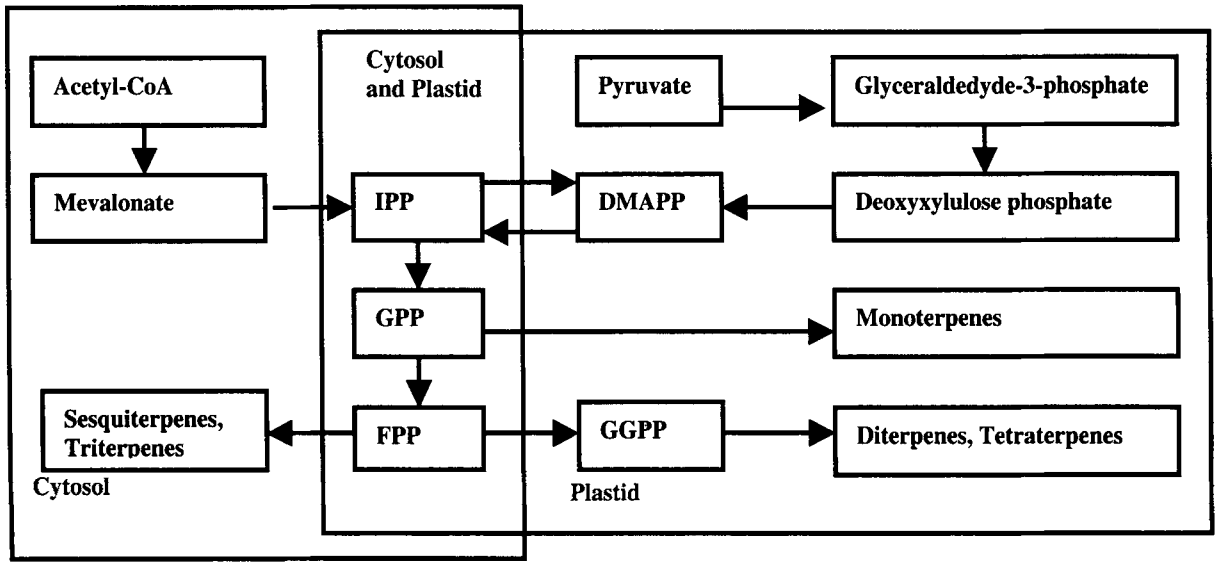

Fig. 3 Intracellular compartmentalization of the mevalonate and mevalonate-independent pathways for the production of isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), and associated terpenoids in higher plants.

The cytosolic pool of IPP, which serves as a precursor of farnesyl diphosphate (FPP) and, ultimately, the sesquiterpenes and triterpenes, is derived from mevalonic acid. The plastidial pool of IPP is derived from the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate, and it provides the precursor of geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) and, ultimately, the monoterpenes, diterpenes, and tetraterpenes. Reactions common to both pathways are enclosed by both boxes, although separate prenyltransferases are responsible for catalyzing these reactions in each compartment.

Great progress in the molecular genetics of terpenoid formation has been made with angiosperms and genes encoding a monoterpene synthase, a sesquiterpene synthase, and a diterpene synthase (Trapp and Croteau 2001). The first terpenoid synthase cDNA was obtained by the reverse genetic approach from grand fir. Gymnosperm terpene synthases resemble in primary sequence their angiosperm counterparts, seven monoterpene synthase genes (Bohlmann *et al.* 1999), three sesquiterpene synthase genes (Bohlmann *et al.* 1998), and two diterpene synthase genes have been isolated and confirmed by functional heterologous expression. All these genes have been obtained from grand fir with the exception of one, the diterpene cyclase taxadiene synthase from yew (*Taxus*) that catalyzes the first committed step in the biosynthesis of the anti-cancer drug Taxol (Wildung and Croteau 1996). The monoterpene synthases and diterpene synthases are encoded as preproteins bearing plastidial transit peptides, whereas the sesquiterpene synthases bear no obvious N-terminal targeting information befitting their cytosolic localization. Tandem arginines near the N terminus of mature monoterpene synthases appear to play a role in the isomerization step required by most enzymes of this class (Williams *et al.* 1998). Although no crystal structures of conifer terpene synthases are yet available, modeling studies based upon the structure of *epi*aristolochene synthase from tobacco (Starks *et al.* 1997) suggest that all terpenoid synthases may share a similar overall folding pattern (Table 1). Terpene synthase cDNAs encode proteins of 550–850 amino acids (Bohlmann *et al.* 1998), in agreement with observed native molecular masses in the 50–100 kDa range. Although conifer monoterpene and diterpene synthases lack significant primary sequence similarity in the transit peptide region, these targeting sequences characteristically contain a high content of serine and threonine residues and a low content of acidic residues (Keegstra *et al.* 1989). Truncation studies with limonene synthase have demonstrated that residues upstream of the tandem arginine element are not required for activity and that the RR motif plays an important role in the isomerization step of

the monoterpene cyclization reaction (Williams *et al.* 1998). Based upon modeling from the crystal structure of aristolochene synthase (Starks *et al.* 1997), investigators found that conifer terpene synthases are composed of two distinct structural domains, a C-terminal active site domain and an N-terminal domain that structurally resembles the catalytic cores of glycosyl hydrolases (Bohlmann *et al.* 1998).

Table 1 Conifer and other selected terpene synthases

Terpene synthase	Species[a]	Gene name	Enzyme name	cDNA name	cDNA Acc #[a]
Abietadiene[b]	A. grandis	ag22	AgggABI	Agggabi	U50765
(E)-α-Bisabolene[c]	A. grandis	ag1	AgfEαBIS	Ag fEαbis	AF006195
(-)-Camphene	A. grandis	ag6	Agg-CAM	Agg-cam	U87910
γ-Humulene	A. grandis	ag5	Agf γHUM	Ag fγ hum	U92267
(-)-4S-Limonene	A. grandis	ag10	Agg-LIM1	Agg-lim	AF006193
Myrcene	A. grandis	ag2	AggMYR	Aggmyr	U87908
(-)-α-Pinene	A. grandis	ag11	Agg-PIN2	Agg-pin2	AF139207
δ-Selinene	A. grandis	ag4	AgfδSEL1	Ag fδsel1	U92266
Taxadiene	T. brevifolia	Tb1	TbggTAX	Tbggtax	U48796
Terpinolene	A. grandis	ag9	AggTEO	Aggteo	AF139206
5-epi-Aristolochene	N. tabacum	TEAS3	NtfeARI3	Ntfeari3	L04680
δ-Cadinene	G. arboreum	CAD1-A	GafδCAD1A	Gafδcad1a	X96429
Casbene	R. communis	cas	RcggCAS	Rcggcas	L32134
ent-Kaurene[b]	A. thaliana	GA2	Atgg-KAU	Atgg-kau	443904
(4S)-Limonene	M. spicata	LMS	Msg-LIM	Msg-lim	L13459
Vetispiradiene	H. muticus	CVS1	HmfVET1	Hmfvet1	U20188

[a]Abbreviations are: Acc #, EMBL Accession number; Species names are: *Abies grandis, Arabidopsis thaliana, Clarkia concinna, Gossypium arboreum, Hyoscyamus muticus, Mentha spicata, Nicotiana tabacum, Ricinus communis, Taxus brevifolia.* [b]Former names for (-)-copalyl diphosphate and *ent*-kaurene sythase were *ent*-kaurene sythase A and *ent*-kaurene sythase B, respectively. [c]Nomenclature architecture is specified as follows: The latin binomial two letter abbreviations are in spaces 1–2. The substrate and product (1–4 letters abbreviation) are in spaces 3–6, consisting of 1–2 letter abbreviation for substrate utilized in bold. The three-letter product abbreviation indicates the major product is an olefin, otherwise the quenching nucleophile is indicated (e.g. ABI, abietadiene synthase); the upper case specifies protein and the lower case specifies cDNA.

The phylogenetic reconstruction based upon amino acid sequence comparison of 33 plant terpene synthases indicates that conifer monoterpene, sesquiterpene, and diterpene synthases are more closely related to each other than they are to their respective, mechanism-based counterparts of angiosperm origin (Bohlmann et al. 1998). Additionally, conifer synthases within each class of the same species are more closely related to each other than they are to the same cyclase type of other conifers. The terpene synthases of gymnosperms and angiosperms are divided into six gene families, designated Tpsa through Tpsf, with the groupings based upon a minimum amino acid sequence identity of 40% (Bohlmann *et al.* 1998). Terpenoids involved in primary metabolism are essential for viability, whereas those derived from secondary metabolism are not. The majority of terpene synthases analyzed to date produce secondary metabolites (natural products) and are classified into three families, Tpsa (sesquiterpene and diterpene synthases from angiosperms), Tpsb (monoterpene synthases from angiosperms of the Lamiaceae), and Tpsd (11 gymnosperm mono-, sesqui-, and diterpene synthases from *A. grandis* and a diterpene synthase, TbggTAX, from *T. brevifolia*). The grouping into a single clade of other Tps families (Tpsc, Tpse, Tpsf) involved in primary metabolism suggests that the bifurcation of terpenoid synthases of primary and secondary metabolism occurred before the separation of angiosperms and gymnosperms (Trapp and Croteau 2001). Furthermore, the pattern of branching between gymnosperm and angiosperm synthases and among specific synthase classes (monoterpene, sesquiterpene, and diterpene) from a common terpene synthase ancestor implies that independent functional specialization occurred after the separation of angiosperm and gymnosperm lineages. That two limonene synthases, MsgLIM and AggLIM, share only 35% identity reflects this independent specialization and indicates that limonene synthase evolved separately in mint and grand fir (Bohlmann et al. 1998). A variety of additional terpene synthase sequences from different gymnosperm species will be necessary to determine the accuracy of the present Tps phylogenetic scheme and its evolutionary significance.

Structural feathers of terpene synthase genomic organization

The examination of terpene synthase genomic (intron/exon) organization generally supports the protein-based phylogenetic evaluation of this enzyme class (Bohlmann *et al.* 1998). Genomic evaluation provides the first model for the history of the plant terpene synthase gene superfamily, including molecular evolutionary events and ancestral lineage. Until recently, the sequences of only a few angiosperm terpene synthase genes (Nt*fepiari*, Rc*ggcas*, Hm*fvet*) had been described, and these revealed very similar overall structure with six positionally conserved introns (Mau and West 1994). The sequences for six coni-

fer terpene synthase genes are now available, including a constitutive and an inducible monoterpene synthase (Ag*glim*, Ag*gpin*), a constitutive and an inducible sesquiterpene synthase, and two diterpene synthases (Ag*ggabi*, Tb*ggtax*). The genomic organization of these conifer terpene synthases was analyzed by alignment with seven defined angiosperm terpene synthases and eight putative terpene synthases from *Arabidopsis*. By examining patterns of intron and exon loss, CDIS domain loss, conservation of intron phase and placement, and conservation of exon size, investigators addressed evolutionary relationships among the plant terpene synthase genes involved in primary and secondary metabolism (Trapp and Croteau 2001). Three classes of terpene synthase genes were established based upon distinct exon/intron patterns, and all conifer synthase genes fell into Class I or Class II. Class I comprises conifer diterpene synthase genes Ag*ggabi* and Tb*ggtax*, and angiosperm synthase genes specifically involved in primary metabolism (At*gg-coppl* and Cc*glin*). Terpene synthase Class I genes contain 11–14 introns and 12–15 exons of characteristic size, including the CDIS domain made up of exons 4, 5, and 6; the first 20 aa of exon 7; and introns 4, 5, and 6. Class II terpene synthases comprise only conifer monoterpene synthases and sesquiterpene synthases and contain 9 introns and 10 exons; introns 1 and 2, and the entire CDIS element, have been lost, including introns 4, 5, and 6. Class III terpene synthases contain only angiosperm monoterpene, sesquiterpene, and diterpene synthases involved in secondary metabolism, and these have 6 introns and 7 exons. Introns 1, 2, 7, 9, and 10 and the CDIS domain have been lost in the Class III type (Trapp and Croteau 2001). The introns found in Class III terpene synthase genes (introns 3, 8, 11–14) have been conserved among all gymnosperm and angiosperm plant terpene synthase genes.

The intron phases of introns 2 through 14 are conserved among gymnosperm and angiosperm terpene synthase genes, and this observation provides a novel means of evaluating the relatedness of genes of this type. Intron phase is defined as the placement of the intron before the first, second, or third nucleotide position of the codon and is referred to as phase 0, 1, and 2, respectively (Trapp and Croteau 2001). The conservation of phases among introns indicates divergent evolutionary events. Conversely, if families of terpene synthase genes evolved by convergent evolution, it is unlikely that the introns would be so precisely placed and intron phase conserved. It is reasonably postulated that the ancestral terpene synthase gene most closely resembles a contemporary gene that contains the largest number of exons and introns because only intron loss would be anticipated. Limited by the sample size of 21 terpene synthase genes, the candidate gene is either At*gg-coppl* or Ag*ggabi*, with At*gg-copp* being most likely because it is involved in primary metabolism. Intron loss and CDIS domain loss data, presented in an evolutionary tree model of intron/exon structure, suggest that all angiosperm terpene synthases involved in secondary metabolic processes evolved from a gymnosperm ancestor that contained 9 introns and 10 exons (Trapp and Croteau 2001). The sesquiterpene synthase bisabolene synthase and the monoterpene synthase linalool synthase gene structures are more similar to conifer diterpene synthase genes, all of which contain the CDIS element.

Pathway organization and evolution

Prior to the availability of any sequence conservation data and based on immunochemical evidence, the suggestion was made that multiple monoterpene synthase genes arose by gene duplication to provide a family of related catalysts for the synthesis of different monoterpene products (Gijzen *et al.* 1992). Limited sequence comparisons between other terpene synthases, based largely on conserved structural features such as the mechanistically relevant DDXXD motif, supported this general notion (Mau and West 1994). The conservation of genomic organization of plant terpene synthases provides further evidence that the terpene synthases from gymnosperms and angiosperms constitute a superfamily of genes derived from a single ancestor. The terpene synthase multigene family tree arose by subsequent duplication, then functional and structural specialization, by evolutionary processes now considered to be quite common (Fryxell 1996). One copy of the duplicated ancestral gene remained conserved in structure and function with little or no intron/exon loss, and this gene may have contemporary descendants in the terpene synthases involved in gibberellin biosynthesis. The second ancestral gene copy diverged in structure and function by adaptive evolutionary processes over millions of years to yield the large multigene superfamily of terpene synthases involved in secondary metabolic pathways. It is plausible that terpene synthase ancestors were functionally less specialized and perhaps able to utilize multiple prenyl diphosphate substrates for the production of multiple terpene types, the specialization into different classes having evolved much later. The evolution of the extant large number of terpene synthase genes provides an example in which many functionally complementary and nonlethal gene duplication and divergence events were retained by natural selection to provide the great diversity in terpene chemistry as the foundation of conifer defense (Trapp and Croteau 2001). Thus, terpene synthase genes evolved to produce new functions that through evolutionary adaptation increased the fitness of the species to defend against predators, pathogens, and herbivores.

An important challenge for the immediate future is to identify and isolate all genes of oleoresin terpenoid biosynthetic pathways. The terpene synthases are presumed to catalyze rate-limiting steps in terpene biosynthesis and thus are obvious targets for molecular genetic manipulation to improve tree resistance. In the biosynthesis of the common resin acid abietic acid in firs and pines, the cyclization product abietadiene is sequentially oxidized at the C^{18} methyl group by two cytochrome P450 hydroxylases and a

soluble aldehyde dehydrogenase to yield the corresponding carboxyl function (Funk and Croteau 1994). Variations on the cyclization scheme can account for the formation of essentially all of the labdane, pimarane, and abietane resin acids of conifers (LaFever *et al.* 1994). Similar biosynthetic steps, involving cytochrome P450 and redox enzymes, are likely involved in the conversion of the cyclization product to todomatuic acid (Bohlmann *et al.* 1998). Genes encoding cytochrome P450 oxygenases and redox enzymes can be isolated based on homology; however, the very large number of these genes makes the sorting of candidates by functional expression of the relevant activities arduous. Furthermore, the extremely large genome size of conifers and their exceedingly long development cycles (Robinson 1999) effectively preclude mutagenesis-based approaches for identification of gene function by phenotype. As an alternative to gene cloning via purification of the target protein, the clustering of secondary metabolic pathway genes may provide rapid access to these sequences. Clustering of fungal sesquiterpene biosynthetic genes for simple and macrocyclic trichothecene production exists in several fungal species (Trapp and Croteau 2001), and three putative sesquiterpene synthase genes, resembling cadinene synthase, vetispiradiene synthase, and *epi*-aristolochene synthase, are located adjacent to each other on chromosome IV of *Arabidopsis* (Aubourg *et al.* 1997). Whether genes encoding enzymes for the oxidative modification of cyclic terpene parent compounds reside in proximity to the corresponding terpene synthases is not presently known.

Resin production and accumulation

Since the first members of terpene were isolated and their structures determined, the study of conifer resin components is entwined with the development of classical organic chemistry and biogenetic theory (Croteau 1998). More recent studies in the context of resin production have focused on those properties most relevant to host defense function such as insect toxicity and solvation properties of turpentine components and on the biological origins of this unique plant secretion. Copious resin production responsible for the killing and encasing of bark beetles and fungal associates is the most important chemical defense of the host in repelling initial invasion. Most conifers rely on some combination of preformed and inducible resin defenses, but there is a fairly clear correlation between the anatomical complexity of the specialized resin secretory structures and the reliance on constitutive defenses (Lewinsohn *et al.* 1991). The compartmentalized structures include: 1) resin cells, which contain oleoresin that are scattered throughout the stems of certain conifer species, including western red cedar (*Thuja plicata*; Cupressaceae); 2) resin blisters, which exhibit a higher degree of anatomical organization in their resin secretory structures, such as California redwood (*Sequoia sempervirens*; Taxodiaceae), true firs, and grand fir (*Abies grandis*; Pinaceae), accumulate oleoresin in the xylem; 3) constricted resin ducts, such as Douglas Fir (*Pseudotsuga menziesii*), western larch (*Larix occidentalis*), and Colorado blue spruce (*Picea pungens*) display an even higher order of organization in which a network of constricted resin passages and ducts reside throughout the trunk; 4) interconnected nonconstricted resin ducts, pine species (*Pinus*) contain the most elaborate network of interconnected nonconstricted resin ducts located throughout the wood and bark (Facchini and Chappell 1992). The longevity of the secretory epithelial cells that produce the oleoresin is also loosely correlated with anatomy; thus, the more complex the resin secretory system, as in pines, the more long-lived are the epithelial cells (Jain 1976).

If a bark beetle severs a resin passage or duct system of a pine or spruce in the process of establishing a gallery, it is often overwhelmed by the rapid and copious exudation of resin, which results in sudden death (Cook and Hain 1988). The efficacy of the process is primarily dependent upon the composition and amount of oleoresin that may flow under pressure to the site of attack by emptying connected resin passages along several meters of the trunk. The response to an individual boring insect depends on attack density and the overall physiological condition of the tree (Gijzen et al. 1993). Only conifer species with substantial constitutive reservoir systems (pines and spruces) can depend upon such preformed resistance as a primary defense strategy (Berryman 1972). A secondary response involving the induced production of resin can often be distinguished in these species, but the amount is small compared with the initial resin flow. Bark beetles occasionally penetrate resin cells and blisters in their excavation; however, the scattered nature of these structures in the trunk limits their utility in primary defense. Species lacking extensive resin ducts or passages (*Abies*, *Tsuga*, *Cedrus*, etc) must rely on induced oleoresin production as a defense against bark beetle infestation, and this localized response is usually part of a more generalized hypersensitive reaction at the site of injury (Berryman 1972). Induced resin production is not carried out by epithelial cells of secretory structures but rather by parenchyma cells surrounding the site of injury. Secondary resin can usually be distinguished from primary resin by chemical composition, and studies with grand fir (*Abies grandis*) have indicated that de novo resin biosynthesis is much the same whether induction results from beetle penetration or other physical trauma, with the level of production reflecting the extent of injury (Lewinsohn *et al.* 1991). Although induced resin production is necessarily a slower process than the immediate flow of stored resin to a wound site, de novo resin production at the site of injury in a healthy response nevertheless can create a formidable barrier to beetle excavation. Conifers, such as firs, that lack an interconnected duct system often respond to wounding by forming traumatic resin ducts, which are normally absent in uninjured tissue (Kuroda and Shimaji 1983). These cyst-like structures accumulate resin but lack epithelial cells. By contrast, pines respond to wounding by forming more resin ducts that are anatomically indistinguishable from normal ducts but are

often improperly called traumatic resin ducts (Kuroda and Shimaji 1983).

Interactions between conifer and bark beetle

The pests and pathogens in conifers include insect defoliators, root feeders, nematodes, and bark boring beetles (*Coleoptera:Scolytidae*) (Schopf 1986). Bark boring beetles are the most destructive agent of coniferous forests worldwide with annual losses in the United States alone exceeding five million board feet of lumber (8). Bark beetle outbreaks are generally episodic and can rapidly spread over hundreds of square miles of forest before abating. This selectivity, wherein a given beetle species will attack only one or two conifer species, reflects the extended period of coevolution between insect and host (Berryman 1972). The biology, behavior, and ecology of bark beetles and their unusual mutualistic relationship with the pathogenic fungi they carry as well as the role of host resin production in resistance against attack have been described for a range of specific beetle-conifer interactions (Berryman 1972). The details of the bark beetle life cycle vary with species and geographic location; however, four major phases can be defined as multiplication, development, dispersal, and concentration (Berryman 1972). In the process of colonization, which has the effect of girdling the tree, the ailing host is also infected with pathogenic fungi carried by the beetles in specialized anatomical structures called mycangia. The larvae hatch, feed in the tunnels that they construct, develop, and then pupate and over winter. In the dispersal phase, young adults emerge from the dead host by boring exit holes and then seek new living hosts by utilizing a variety of visual, tactile, and olfactory cues (Wood 1982). Once a suitable host is located, often a physically damaged or physiologically compromised tree, pioneer beetles signal for mass attack in an attempt to overcome host defenses (Wood 1982). This concentrated attack often results in the death of the tree in which the annual cycle of multiplication, development, dispersal, and concentration is repeated.

The bark beetle–microbe-tree ecosystem has been described as a bark beetle microbial symbiosis in which bark beetles associate in a generally mutualistic relationship with a variety of microorganisms (fungi, yeasts, bacteria, and protozoans) borne on and in their bodies (Lewinsohn *et al.* 1999). Pathogenic fungi are often designated as blue- or black-staining because they typically discolor the infected wood and reduce its lumber value. All bark beetle species appear to vector pathogenic fungi, the most common associative types belonging to the genera *Ophiostoma* (formerly *Ceratocystis*), *Leptographium*, *Graphium*, and *Trichosporium*. Vectored yeasts may serve as food sources for beetles and their broods (Lewinsohn *et al.* 1999). The volatile monoterpene constituents of conifer oleoresin serve diverse roles in bark beetle chemical ecology, including host recognition and selection; pheromone signaling, which directs beetle aggregation and colonization; and tritrophic level interactions that involve signaling of beetle predators and parasitoids (Wood 1982). In spite of their toxicity toward bark beetles, resin monoterpenes provide a species-specific chemical signature and thus function as olfactory cues in host location (Wood 1982). The monoterpene air plume derived from freshly exposed oleoresin is generally attractive to pioneer beetles, as are the emissions from physiologically compromised individuals (Lewinsohn *et al.* 1999). Such sites lead to the aggregation and colonization phases of host attack. The essential advantage of a focused attack is that the host is unable to respond to the sheer number of invaders. Many bark beetle species synthesize aggregation pheromones from turpentine components of host oleoresin.

Although the involvement of bark beetles in the production of pheromones is clear, the biosynthetic origin of these signaling compounds is still at issue. Some fungal symbionts and insect gut microorganisms are able to oxidize the host monoterpenes to the corresponding pheromones (Lewinsohn *et al.* 1991). Francke & Vite (1983) have hypothesized that the oxidative modification of host monoterpenes by bark beetles may have arisen as a detoxification mechanism. Insect predators and parasitoids of bark beetles also respond to bark beetle pheromones and to conifer monoterpenes, but the specificity of these responses is not well understood, in part because these insects tend to feed on a wide range of prey and may exploit a broad range of chemical signals (Payne 1989). An illustrative example of these complex interactions is provided by three trophic levels of Norway spruce (*Picea abies*), the European spruce bark beetle (*Dendroctonus micans*), and the predatory beetle (*Rhizophagus grandis*). Within this interaction, resin monoterpenes attract both bark beetles and predator beetles to mediate host location and bark beetle aggregation. Although it is paradoxical that bark beetles are attracted to and exploit monoterpenes, the benefits to the beetle of utilizing specific semiochemical cues to locate a suitable host must outweigh the disadvantages. These interactions illustrate the complexity of the evolutionary relationships between conifer host and insect herbivores, causing selection over many generations to produce bark beetles that are highly adapted to a particular host and conifers that produce highly diverse chemical defenses (Gijzen *et al.* 1993). It is also important to note that oleoresin also provides the basis of complex interactions with numerous other conifer pests and pathogens, including insect defoliators, root feeders, nematodes, and even mammalian and avian herbivores (Schopf 1986, Trapp and Croteau 2001).

Engineering strategies to improve conifer defenses

The current understanding of what constitutes superior traits at the molecular level is still quite rudimentary and lags significantly behind other fields of Agro-biotechnology (Robinson 1999). An important challenge is to rapidly ac-

quire genes underpinning desirable traits and to do so without the immediate benefit of a highly revealing conifer genome project (Sederoff 1999). In addition to the long-term commitment required to make forest biotechnology a commercial reality (Robinson 1999), legitimate environmental concerns will also need to be addressed, and public education will be necessary to overcome the adverse perception of plant genetic engineering, especially when applied on the large-scale in the forest setting (James 1997). The ecological interactions between conifer hosts, pathogens, and bark beetles and their predators and parasitoids that are mediated by oleoresin terpenoids are exceedingly complex, yet they offer several possible avenues for improving tree resistance by manipulation of oleoresin composition. Terpene synthases are conceptually attractive and obvious candidates for this purpose, and the recent cloning of a number of terpene synthase cDNAs now offers a bio-rational approach for improving conifer defenses by altering not only the mix of constitutive and inducible oleoresin but also the yield and composition of oleoresin itself via gene transfer technologies.

Understanding the molecular genetics, organization, and regulation of constitutive and inducible oleoresin formation underlies the ability to design protective and management strategies for providing sustainable forest products. The approaches to engineered manipulation of oleoresin formation include: (1) improving the speed and level of the defense response at the critical early attack stages; (2) increasing the concentration of resin components that are particularly toxic to invaders by modulating promoter strength and copy number of extant genes, and by introducing new defense genes; (3) altering oleoresin chemistry to disguise the host and thereby confuse host selection; (4) promoting tritrophic level interactions through improved signaling to foster bark beetle predation or parasitism; (5) engineering trees to produce disperal pheromones or produce hormone analogs to disrupt insect reproduction and development. There are still many aspects of oleoresin-based conifer defense that remain unexplored. Cell-specific constitutive promoters for controlling primary resin formation or wound-specific promoters for controlling attack dependent defense genes have not been defined, nor have transcription factors involved in these processes been described. Very little is known about the signaling cues and downstream cascade pathways that mediate the communication between host and pest or pathogen (Trapp and Croteau 2001). Information on the determinants of substrate specificity and product outcome would permit the redesign of these catalysts. Advances in each of these areas can be expected to lead to new strategies for tree protection.

Conclusions

The prospects for genetically engineering conifer defenses to improve resistance promise a bright future for forest biotechnology. Although the agricultural domestication of forest species is still in its infancy, forests are internationally recognized as one of the most important of natural resources. Genetic improvement in agro-forestry has relied primarily on conventional breeding programs to alter characteristics such as resistance to pests and pathogens, growth rate and form, volume and yield, and quality of end product lumber and paper pulp. However, this approach is clearly limited by the generation time required for selection of improved trees. It is sobering to realize that even the most advanced forest tree-breeding programs are in their third generation. The application of molecular genetics and biotechnological techniques based on gene transfer to engineer conifers presented great progress. More recently, antisense technologies are being applied to forest species and specific means of eliminating forest destruction caused by insect pests and their microbial symbionts will be established. The promotion of biological diversity with minimum impact on the functioning equilibrium of the ecosystem is exciting to scientists. Most insects represent a far greater diversity of species and impact on the structure and function of the complex forest ecosystem. Biotechnologically based pest management strategies founded on natural defense mechanisms need to be explored incrementally in both type and scale. Improving defenses in conifer will increase the production of forest industries and benefit to the environment all over the world.

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